APPENDIX 1

Protocol #2009681-404
Protocol Amendment Form – EPA (Template Form: G-AMEND-PR-EPA)

October 28, 2020

PROTOCOL #2009681-404

A GLP VIRUCIDAL EFFICACY EVALUATION OF ONE HARD SURFACE DISINFECTANT SUBSTANCE

Prepared for:

SOLVAY USA (SPONSOR)
350 George Patterson Boulevard
Bristol, Pennsylvania 19007

Prepared by:

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1.0 TITLE: **A GLP VIRUCIDAL EFFICACY EVALUATION OF ONE HARD SURFACE DISINFECTANT SUBSTANCE**

2.0 SPONSOR: **SOLVAY USA**
350 George Patterson Boulevard
Bristol, Pennsylvania 19007

3.0 TESTING FACILITY: **BIOSCIENCE LABORATORIES, INC.**
1755 South 19th Avenue
Bozeman, Montana 59718

4.0 STUDY DIRECTOR: Volha Teagle, Ph.D.

5.0 PROPOSED EXPERIMENTAL START DATE: 11/03/2020

The experimental start time as specified in OCSPP 810.2000 does not affect the scientific outcome of this study and will not be reported in the Final Report unless requested by the Sponsor prior to the start of the study.

6.0 PROPOSED EXPERIMENTAL COMPLETION DATE: 12/22/2020

The experimental end time as specified in OCSPP 810.2000 does not affect the scientific outcome of this study and will not be reported in the Final Report unless requested by the Sponsor prior to the start of the study.

7.0 PURPOSE:

The purpose of this study is to evaluate the virucidal efficacy of one disinfectant test substance when challenged with multiple virus strains. Testing will be based upon methods described as specified in the American Society for Test Materials (ASTM) test methods designated E1053-20, *Standard Test Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surface*, as specified in U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, *OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces* (February 2018), and *OCSPP 810.2000 : General Considerations for Testing Public Health Antimicrobial Pesticides* (February 2018). All testing will be performed in accordance with Good Laboratory Practices, as specified in 40 CFR 160. The Sponsor is required to provide a Certificate of Analysis for the chemical characterization of the test batches. The Certificate will be appended to the final report.

8.0 SCOPE:

This study will evaluate the virucidal efficacy of one disinfectant test substance, when used on dry, non-porous, inanimate surfaces. The test substance will be evaluated in the presence of an Organic Soil Load (OSL) versus Influenza A H1N1 strain A/WS/33 (ATCC #VR-1520), Feline Calicivirus strain F9 (FCV; ATCC #VR-782), a surrogate for Human Norovirus, Human Respiratory Syncytial Virus, strain Long (HRSV, ATCC #VR-26), Coronavirus strain 229E (ATCC #VR-740), and SARS-CoV-2 strain USA-WA1/2020 (BEI Resources # NR52281). Two batches of the test substance will be used for testing against Influenza A, FCV, HRSV, and Coronavirus 229E. Three batches of the test substance will be used for testing against SARS-CoV-2. The disinfectant test substance will be provided as a ready to use formulation. A challenge suspension will be used to inoculate the bottom part of 100 mm X 15 mm glass Petri Dish carriers to yield a minimum of $10^{4.8}$ viruses per carrier following drying. After drying, each carrier will be exposed to 2.0 mL of the test substance at room temperature for exposure time. Following the timed exposure, the neutralizer appropriate for the test substance

will be added to the carrier. An aliquot of the neutralized suspension will be serially diluted in medium and assayed for the presence of viable viruses using the cell culture susceptible to the virus. The viral titers will be determined using a 50% tissue culture infectious dose ($TCID_{50}$) calculation -- the Quantal test (Spearman-Kärber Method).

9.0 JUSTIFICATION FOR THE SELECTION OF THE TEST SYSTEM:

The Sponsor has requested a disinfectant label claim for human Norovirus, Influenza A virus, HRSV, Coronavirus 229E, and SARS-CoV-2. EPA requires all Test Systems claimed on the label to be tested in accordance with EPA 810 guidelines. FCV (ATCC #VR-782) will be used as a surrogate for human Norovirus. This protocol aligns with the requirements for virucidal testing and virucidal testing using surrogate viruses outlined in 810.2000 and 810.2200.

10.0 TEST SUBSTANCE:

The test substance to be evaluated will be provided to the Testing Facility by the Study Sponsor, complete with appropriate documentation. Responsibility for the determination of the identity, strength, purity, composition, stability, and solubility of the test substance, as well as the retention of the test substance, rests with the Sponsor.

Test Substance:	DV 5-26762
Active Ingredients:	Alkyl (50%C14, 40%C12, 10%C16) dimethylbenzyl ammonium chloride (C12-C16) [ADBAC] CAS RN: 68424-85-1 Didecyldimethylammonium chloride / [DDAC] CAS RN: 7173-51-5
Batch Number 1	
Lot Number:	S1528-205-27D
Manufacture Date:	August 27, 2020
Expiration Date:	August 27, 2022
Batch Number 2	
Lot Number:	S1528-205-18D
Manufacture Date:	August 26, 2020
Expiration Date:	August 26, 2022
Batch Number 3	
Lot Number:	S1528-205-09D
Manufacture Date:	August 25, 2020
Expiration Date:	August 25, 2022

11.0 TEST CONDITIONS:

	Influenza A	FCV	HRSV	Coronavirus 229E	SARS-CoV-2
Exposure Time:	5 minutes	10 minutes	5 minutes	5 minutes	5 minutes
# of Batches:	2	2	2	2	3
# of Carriers per Batch:	1	2	1	1	1
Exposure Temperature:	18 °C to 25 °C				
Test Dilution:	None				
Exposure Humidity:	Ambient				
Soil Load:	5% (v/v) Heat-inactivated Fetal Bovine Serum (FBS)				

12.0 EQUIPMENT:

12.1 CO_2 Incubator, Temperature Range 37 °C ± 2 °C with 4 % to 6% CO_2
12.2 CO_2 Incubator, Temperature Range 33 °C ± 2 °C with 4 % to 6% CO_2
12.3 CO_2 Incubator, Temperature Range 35 °C ± 2 °C with 4 % to 6% CO_2
12.4 Thermometers
12.5 Portable Pipetter
12.6 Continuously Adjustable Pipettes, 100 µL – 1000 µL Capacity
12.7 Continuously Adjustable Pipettes, 20 µL – 200 µL Capacity
12.8 Inverted Compound Microscope
12.9 Laminar Flow Biological Safety Cabinet
12.10 Waste Pan
12.11 Calibrated Minute/Second Timers
12.12 Hygrometer

13.0 SUPPLIES:

13.1 Sterile Disposable Pipettes
13.2 Sterile Polystyrene Test Tubes
13.3 Sterile Universal 1.0 mL and 0.2 mL Pipette Tips
13.4 Powder-Free Gloves
13.5 Sterile Tissue Culture Treated 24-Well Plates
13.6 Viral Suspension(s)
13.7 Sterile Flasks
13.8 Sterile 50 mL Centrifuge Tubes
13.9 Sterile Pipette Reservoir
13.10 Non-Sterile Waste Beaker for discarded tips, etc.
13.11 Sterile Cell Scrapers
13.12 Sterile Glass Petri Dishes

14.0 MEDIA:

14.1 1X Eagle's Minimum Essential Medium (EMEM), or other appropriate medium
14.2 Growth Medium: EMEM with 10% non-heat inactivated FBS or Horse Serum, 1% penicillin-streptomycin-amphotericin B, 1% of L-Glutamine (when necessary)
14.3 Maintenance Medium: EMEM with 2% FBS, 1% penicillin-streptomycin-amphotericin B, 1% of L-Glutamine (when necessary); EMEM with 2% Horse Serum, 1% penicillin-streptomycin-amphotericin B, 1% of L-Glutamine (when necessary); EMEM with 1% penicillin-streptomycin-amphotericin B, 1% of L-Glutamine (when necessary), and 1µg/mL TPCK-Treated Trypsin
14.4 Trypsin/EDTA for cell culture maintenance
14.5 TPCK-Treated Trypsin
14.6 Antibiotics for medium
14.7 Appropriate Neutralizer
14.8 Fetal Bovine Serum (FBS)

15.0 ORGANIC SOIL LOAD:

5% (v/v) Heat-inactivated Fetal Bovine Serum (FBS).

16.0 DILUENT:

No diluent will be used.

17.0 CHALLENGE VIRAL STRAIN:

17.1 Influenza A H1N1 strain A/WS/33 (ATCC #VR-1520)
17.2 Feline Calicivirus strain F9 a surrogate for Human Norovirus (FCV; ATCC #VR-782)
17.3 Human Respiratory Syncytial Virus, strain Long (HRSV; ATCC #VR-26)
17.4 Coronavirus strain 229E (ATCC #VR-740)
17.5 SARS-CoV-2 strain USA-WA1/2020 (BEI Resources # NR52281).
ATCC = American Type Culture Collection
BEI Resources = Biological and Emerging Infections Resources Program (BEI Resources) National Institute of Allergy and Infectious Diseases (NIAID).

18.0 HOST CELLS:

18.1 MDCK (ATCC #CCL-34, Madin-Darby Canine Kidney cells, epithelial)
18.2 CRFK (ATCC #CCL-94; Crandell-Reese Feline Kidney cells, epithelial)
18.3 Hep-2 (ATCC #CCL-23, HeLa contaminant, cervical adenocarcinoma, epithelial)
18.4 MRC-5 (ATCC #CCL-171; human lung fibroblasts)
18.5 Vero76[Vero6] (ATCC #CRL-1586; green monkey kidney cells, epithelial)

19.0 HOST CELL PREPARATION:

The cell lines will be maintained as monolayers in disposable cell culture labware and will be used for testing of each virus. Cell monolayers will be allowed to grow for less than 48 hours until approximately 80% to 90% confluent. Growth Medium (GM) will be replaced with Maintenance Medium (MM).

20.0 TEST VIRUS PREPARATION:

The viral suspensions used for this study will originate from BSLI high titer virus stock. On the day of use, heat inactivated FBS will be added to the final concentration of 5%.

21.0 TEST VIRUS IDENTIFICATION:

Cytotoxic viruses will be identified via Cytopathic Effect (CPE) in susceptible cell cultures using inverted compound microscope.

22.0 TEST SUBSTANCE PREPARATION:

The Test Substance will be tested in compliance with the EPA 810.2000 Lower Certified Limit policy. A Certificate of Analysis will be appended to the final report.

23.0 TEST PROCEDURE:

23.1 Preparation of Carriers

Sterilized glass Petri plates (100 mm x 15 mm) will be used as the carriers for this evaluation.

23.2 Contamination of Carriers

23.2.1 A 0.2 mL aliquot of the prepared virus suspension will be transferred to the bottom inside surface of a sterilized 100 mm x 15 mm glass Petri plate carrier. A sterile cell scraper will be used to spread the inoculum uniformly.

23.2.2 The virus suspension(s) will be air-dried at room temperature until visibly dry. Drying conditions (time, temperature, and relative humidity) will be documented.

23.2.3 One carrier per batch of the test substance will be used for Influenza A H1N1, HRSV, Coronavirus 229, and SARS-CoV-2 in accordance with the EPA *OCSPP 810.2200*.

23.2.4 Two carriers per batch will be used for FCV surrogate virus in accordance with the EPA *OCSPP 810.2200*.

23.3 Test

23.3.1 After the inoculated carrier(s) has dried, the carrier(s) will be treated with 2.0 mL of the designated batch of the test substance. The carrier(s) will be exposed to the test substance at ambient temperature for the specified for each virus exposure time, timed using a calibrated minute/second timer. Timing will commence after the liquid substance is spread over the entire surface of the contaminated carrier(s). The treated carrier(s) will be kept undisturbed for the duration of the contact time. Test conditions (time, temperature, and relative humidity) will be documented.

23.3.2 After the exposure time has elapsed, the appropriate amount of the neutralizer (18 mL) will be added to the Petri dish and the virus test substance mixture will be scraped from the surface of the carrier using a sterile cell scraper. Subsequent 10-fold dilutions will be made in MM and plated in four replicates.

23.3.3 Plate Recovery Control. One carrier will be used for the virus recovery control. The test virus will be dried as described in Section 23.2.1 and 23.2.2. A total of 2.0 mL of MM will be added to the contaminated carrier. The carrier will be exposed to MM at ambient temperature for the specified for each virus exposure time, timed using a calibrated minute/second timer. The appropriate neutralizer will be added to the carriers and the virus will be scraped from the surface. Subsequent 10-fold dilutions will be made in MM and plated in four replicates.

23.3.4 Virus Stock Titer. The test virus will be diluted 10-fold in MM. Each dilution will be plated in four replicates.

23.3.5 Neutralization and Cytotoxicity Controls. A 0.2 mL aliquot of medium will be transferred to the bottom inside surface of a sterilized 100 mm x 15 mm glass Petri plate carrier. A sterile cell scraper will be used to spread the medium uniformly. The medium will be air-dried at room temperature until visibly dry. After drying, the carrier will be treated as described in Section 23.3.1. After the exposure time has elapsed, the appropriate neutralizer will be dispensed into the carriers. The neutralized liquid will be used for the Neutralization control (virus added) and Cytotoxicity Control (no virus added). The Neutralization Control will receive an aliquot of a test virus, followed by exposure for at least the specified test exposure time. Subsequent 10-fold dilutions will be made in MM and plated in four replicates. The Cytotoxicity Control will receive no virus, will be diluted (10-fold) in MM, and plated in four replicates.

23.3.6 Virus Control. A dilution of the test virus will be added to a Neutralizer and exposed for at least the exposure time. Subsequent 10-fold dilutions will be made in MM and plated in four replicates.

23.3.7 Neutralizer Cytotoxicity Control. Neutralizing solution will be plated onto cell cultures in at least 4 wells

23.3.8 Cell Culture Control. Intact cell culture monolayers will serve as the control of cell culture viability. The Growth Medium will be replaced by MM in all cell culture control wells (minimum four wells).

23.3.9 The plates will be incubated for 5 to 14 days at in a CO2 incubator at the temperature

appropriate for each virus.

23.3.10 Evaluation of Virus Recovery. Cytopathic/cytotoxic effect will be monitored using an inverted compound microscope.

24.0 CALCULATIONS:

24.1 Viral titers will be expressed as -Log_{10} of the 50% titration end point for infectivity. To calculate the viral titer, a 50% tissue culture infectious dose ($TCID_{50}$) calculation - the Quantal test (Spearman-Kärber Method) - will be applied.

Negative Log of $TCID_{50}$/mL = -log of 1st dilution assayed – [((Σ of % mortality at each dilution/100) – 0.5) × (log of dilution)]

24.2 Virus recoveries will be presented per assay volume and per carrier inoculum volume.

TCID50/carrier = (Antilog of $TCID_{50}$/mL) ×0.2 = Log_{10}/carrier

24.3 The Log_{10} and percent (%) of infectivity reductions will be calculated as follows:

$$\% \text{ Reduction} = \left[1 - \frac{\text{TCID}_{50}\ test}{\text{TCID}_{50}\ plate\ recovery\ control}\right] \times 100$$

Log_{10} Reduction = (Log_{10} $TCID_{50}$ of the Plate Recovery Control) - (Log_{10} $TCID_{50}$ of the Test)

25.0 TEST ACCEPTANCE CRITERIA:

A valid test requires: 1) at least 4.8 log_{10} of $TCID_{50}$ per carrier will be recovered from the Plate Recovery Control; 2) the virus titer from the Plate Recovery Control should be sufficient to show at least 3 log_{10} reduction above the cytotoxicity level (*e.g.*, 5.5 log_{10} Plate Recovery – 2.5 log_{10} Cytotoxicity = 3.00 log_{10} Reduction); 3) cells in the Cell Control wells be viable and attached to the bottom of the well; 4) the medium be free of contamination in all wells of the plate; 5) when cytotoxicity is evident, at least a 3 log_{10} reduction in titer be demonstrated beyond the cytotoxic level; 6) the test substance be fully neutralized, so the difference between the test virus titer of Virus Stock and Neutralization Control does not exceed 1.0 log_{10}.

26.0 STATISTICAL ANALYSIS:

The Quantal test (Spearman-Kärber Method) will be applied to calculate virus titer. No control of bias will be performed.

27.0 PROTOCOL DEVIATIONS AND AMENDMENTS:

Amendments to the approved protocol and the reasons will be documented, signed and dated by the Study Director and Sponsor, and maintained with the protocol per GLP 160.120(15) (b) and current BSLI Standard Operating Procedures. Deviations will be documented by the Study Director, signed and dated and maintained with the protocol per GLP 160.120(15) (b) and current BSLI Standard Operating Procedures.

28.0 FINAL REPORT:

A final report will be prepared by BioScience Laboratories, Inc., describing the results of the study in a clear and concise manner. The final report will include all items required by 40 CFR Part 160.185.

29.0 EXCEPTIONAL CONDITIONS:

The Sponsor will be notified by telephone, email, and/or letter of any exceptions encountered in this study. The exceptional conditions or occurrences will be detailed in full and formally recorded. Exceptional conditions

that occur and are not addressed in this Protocol will be subject to Out-of-Scope charges (See Proposal/Contract).

30.0 LIABILITY AND INDEMNIFICATION:

The Testing Facility's liability to the Study Sponsor under this Protocol shall be limited to the price of this evaluation. The Study Sponsor shall be responsible to Study Participants (when applicable) and to other third parties for the fitness of the test substance for use as defined in the Study Protocol.

31.0 REFERENCES:

31.1 ASTM E1053-20, *Standard Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces*

31.2 U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, *OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces-Guidance for Efficacy Testing* (February 2018).

31.3 U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, *OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides* (February 2018).

32.0 DOCUMENTATION AND RECORD-KEEPING:

All documentation and records will be compiled, analyzed, and retained by BioScience Laboratories, Inc., at its facility in Bozeman, Montana. All raw data for this study will be retained in safe storage for the life of the substance registration with the EPA, or as specified by the Sponsor.

33.0 TEST SUBSTANCE DISPOSITION:

It is the responsibility of the Sponsor to retain a sample of the test substance(s) for future audit or evaluation. All unused test material will be disposed of following study completion, unless otherwise indicated by the Sponsor prior to initiation of the study.

34.0 QUALITY ASSURANCE AUDITS:

Quality Assurance (QA) will conduct in-phase audits of critical processes in testing at least once and advise the Study Director and Management of the outcome. On completion of testing, the QA will perform an audit of the data and of the Final Report in its entirety.

35.0 ACCEPTANCE:

A GLP VIRUCIDAL EFFICACY EVALUATION OF ONE HARD SURFACE DISINFECTANT SUBSTANCE

ACCEPTED BY: BIOSCIENCE LABORATORIES, INC. (TESTING FACILITY)
1755 South 19th Avenue
Bozeman, Montana 59718

Study Director: _____ 11-02-2020
Volha Teagle, Ph.D. Date of Study Initiation

ACCEPTED BY: SOLVAY USA (SPONSOR)
350 George Patterson Boulevard
Bristol, Pennsylvania 19007

_____ November 2, 2020
Representative Date

Healthcare & Innovation Manager - Microbiology
Title

PROTOCOL AMENDMENT FORM - EPA

DATE: 12-03-2020 **AMENDMENT NUMBER:** 01

PROTOCOL NUMBER: 2009681-404

SPONSOR: SOLVAY USA

PROTOCOL TITLE: A GLP VIRUCIDAL EFFICACY EVALUATION OF ONE HARD SURFACE DISINFECTANT SUBSTANCE

REASON FOR CHANGE(S): The Sponsor requested to exclude SARS-CoV-2 virus strain USA-WA1/2020 (BEI Resources # NR52281) and the Test substance, DV 5-26762, Batch #3 Lot #S1528-205-09D from testing.

The Sponsor also requested to issue two Final Reports for this study. Final Report # 2009681-404 A will present results for Influenza A H1N1 (ATCC # VR-1520), Human Respiratory Syncytial Virus (ATCC # VR-26), and Coronavirus 229E (ATCC #VR-740). Final Report # 2009681-404 B will present the results for Feline Calicivirus (ATCC #VR-782).

CHANGE(S):

SARS-CoV-2 virus strain USA-WA1/2020 (BEI Resources # NR52281) and the Test substance, DV 5-26762, Batch #3 Lot #S1528-205-09D will not be used in testing for the study # 2009681-404.
Two Final Reports #2009681-404 A and 2009681-404 B will be issued for this study. Final Report # 2009681-404 A will present results for Influenza A H1N1 (ATCC # VR-1520), Human Respiratory Syncytial Virus (ATCC # VR-26), and Coronavirus 229E (ATCC #VR-740). Final Report # 2009681-404 B will present the results for Feline Calicivirus (ATCC #VR-782).

APPROVALS:

SPONSOR	12-03-2020 DATE
STUDY DIRECTOR	12-03-2020 DATE

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